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Investigations of metabolic precursors to hemoglobin and DNA adducts of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone

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Levels of DNA and/or hemoglobin pyridyloxobutylation were compared in A/J mice or F344 rats treated with a single dose of [5-^{3}H]4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone ([5-^{3}H]NNK), [5-^{3}H]4-hydroxy-1-(3-pyridyl)-1-butanone ([5-^{3}H]-4-HPB) or [5-^{3}H]4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone ([5-^{3}H]NNKOAc), a compound that generates the proposed pyridyloxobutylating agent *in situ* upon esterase hydrolysis. The lung and liver DNA samples isolated from A/J mice treated with the various compounds were subjected to acid hydrolysis and the hydrolysates were analyzed for the presence of [5-^{3}H]4-HPB. No detectable levels were found in the lung DNA isolated from [5-^{3}H]4-HPB-treated animals, whereas significant amounts of [5-^{3}H]4-HPB were released from lung and liver DNA isolated from [5-^{3}H]NNK- and [5-^{3}H]NNKOAc-treated mice. The levels of total binding and [5-^{3}H]4-HPB released from the globin isolated from these animals showed a similar trend. That is, low binding levels were detected in the globin isolated from [5-^{3}H]4-HPB-treated animals and significantly higher levels of binding were detected in the globin from the [5-^{3}H]NNKOAc- and [5-^{3}H]NNK-treated animals. Comparable findings were obtained in the rat experiments. These studies clearly demonstrate that methyl hydroxylation of NNK leads to a species that is capable of reacting covalently with nucleophiles in DNA and protein. Thus, the levels of 4-HPB released from DNA and globin can be attributed to the activation of NNK and not to the direct binding of 4-HPB.

Introduction

The tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK*) is a potent carcinogen in laboratory animals (1,2). The observation of relatively high concentrations of this nitrosamine in tobacco products (3) has led to the proposal that NNK plays a role in tobacco-related cancers in humans. NNK is bioactivated to several reactive species capable of binding to hemoglobin and DNA (4,5). One possible activation pathway involves hydroxylation of the methyl group to yield 4-(hydroxymethylnitrosamino)-1-(3-pyridyl)-1-butanone. This metabolite is believed to decompose to 4-oxo-4-(3-pyridyl)-1-butanediazohydroxide upon the release of formaldehyde (Figure 1). This diazohydroxide can either react with water to produce 4-hydroxy-1-(3-pyridyl)-1-butanone (4-HPB) or alkylate cellular nucleophiles in proteins and DNA as demonstrated by the incorporation of tritium into DNA and hemoglobin isolated from rats treated with pyridyl-tritiated NNK ([5-^{3}H]-NNK) (4,5). The chemical nature of these adducts is unknown. In the case of DNA, the adducts are unstable and >50% of the bound radioactivity is released as labeled 4-HPB upon neutral thermal or acid hydrolysis of the DNA (5). Some of the globin adducts are also unstable and decompose to 4-HPB upon base treatment. These unstable adducts account for 15–40% of the radioactivity bound to the globin (4,6). These same adducts are also observed in animals treated with *N'*-nitrosonornicotine (NNN), another tobacco-specific nitrosamine (4,5).

These studies demonstrate that α-hydroxylation of NNK generates a reactive species capable of interacting covalently with cellular nucleophiles. Based on this proposal, a dosimetry assay was developed to measure the levels of 4-HPB released from hemoglobin of tobacco users (7). This assay assesses human exposure levels to NNK and NNN and an individual's ability to activate these carcinogens. However, 4-HPB is a metabolite of NNK and NNN (8) and may also be present in tobacco products. It is possible that 4-HPB itself interacts with DNA and hemoglobin in a reaction that is reversible under the hydrolysis conditions. In order to determine whether 4-HPB released from hemoglobin and DNA is derived from an adduct(s) formed via NNK activation or resulted from direct binding of 4-HPB to these macromolecules, we measured the levels of 4-HPB released from DNA and hemoglobin isolated from rodents treated with [5-^{3}H]-NNK, [5-^{3}H]4-HPB, or [5-^{3}H]4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone (NNKOAc), the acetylated hydroxymethyl metabolite of NNK. This last compound will generate the reactive pyridyloxobutylating agent *in situ* upon esterase hydrolysis

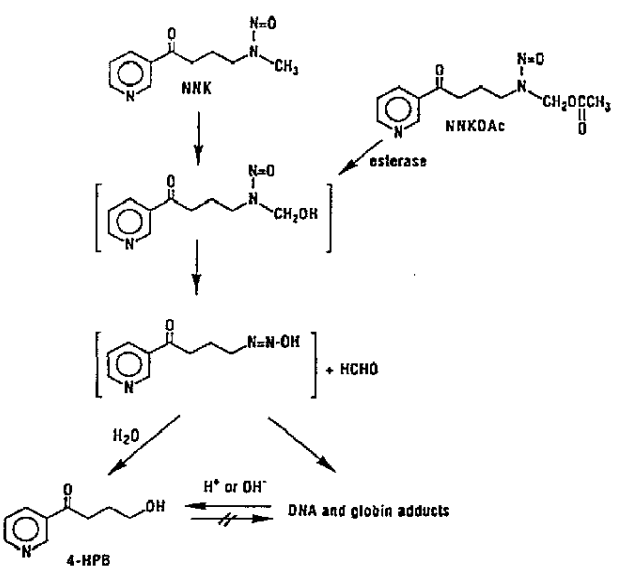

Fig. 1. Proposed activation pathway of NNK to pyridyloxobutylating intermediates.

*Abbreviations: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; 4-HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone; NNN, N'-nitrosonornicotine; NNKOAc, 4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone; myosmine, 3-(3,4-dihydro-2H-pyrrol-5-yl)pyridine; 3-HPB, 3-hydroxy-1-(3-pyridyl)-1-butanone; AcPB, 4-acetoxy-1-(3-pyridyl)-1-butanone; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol.

(Figure 1) (9). We chose to address these questions in the animal model systems previously used to gain mechanistic information about the carcinogenic activity of NNK (1,2).

Materials and methods

Chemicals

[5-^{3}H]NNK (1.2 Ci/mmol; purity, 98%) and [5-^{3}H]3-(3,4-dihydro-2H-pyrrol-5-yl)pyridine ([5-^{3}H]myosmine) (0.80 Ci/mmol; purity, 97%) were obtained from Chemsyn Science Laboratories (Lenexa, KS). Their purities were confirmed by HPLC analysis. They were diluted as described with unlabeled synthetic NNK (10) or myosmine (11). [5-^{3}H]4-HPB used in the rat experiments was synthesized as previously described (4), whereas that used in the mouse experiments was synthesized as described below. Unlabeled 4-HPB and 3-hydroxy-1-(3-pyridyl)-1-butanone (3-HPB) were prepared as previously reported (10,12).

[5-^{3}H]4-(Acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone. The dihydrochloride salt of [5-^{3}H]4-amino-1-(3-pyridyl)-1-butanone was obtained by dissolving [5-^{3}H]myosmine (701 mg, 4.8 mmol, 481 mCi) in 1 N HCl (18 ml). After stirring at room temperature for 2 h, the solution was concentrated to dryness using absolute ethanol. The resulting light brown solid was dissolved in glacial acetic acid (6 ml) with gentle heating (35–40°C). Likewise, paraformaldehyde (153 mg, 5.5 mmol) was dissolved in glacial acetic acid with heating. The paraformaldehyde solution was added to the amine solution and the mixture was cooled to room temperature. After 18 h, the reaction mixture was placed in an ice bath and an aqueous solution (4 ml) of $NaNO_2$ (563 mg, 8.2 mmol) was added dropwise. After the solution was stirred at room temperature for 1.5 h, 50 ml CH_2Cl_2 was added and the aqueous layer was neutralized with a saturated $NaHCO_3$ solution. The CH_2Cl_2 layer was removed and the aqueous phase was extracted three more times with 50 ml CH_2Cl_2. The organic extracts were combined, washed with saturated $NaHCO_3$ solution, dried over anhydrous Na_2SO_4, and filtered. The solution was concentrated to 1 ml and applied to a flash chromatography column (Florisil, 20 g) in CH_2Cl_2. This purification step was done immediately to remove contaminants capable of decomposing the product. The column was first eluted with CH_2Cl_2 (500 ml), then 10% ethyl acetate in CH_2Cl_2 (500 ml), and finally 20% ethyl acetate in CH_2Cl_2 (500 ml). Fractions (20 ml) were collected. The desired product eluted in the 20% ethyl acetate fractions. The fractions containing product (as judged by silica TLC; mobile phase, ethyl acetate) were combined, dried with anhydrous Na_2SO_4, filtered and concentrated to dryness. The residue was redissolved in 50 ml dry CH_2Cl_2 and stored at −20°C under nitrogen. When the crude reaction mixture was stored in this manner, [5-^{3}H]NNKOAc was radiochemically stable for several months. [5-^{3}H]NNKOAc was further purified on a semipreparative silica HPLC column (Whatman Partisil 10 Magnum 9 column, 50 cm) eluting with a linear gradient from 100% $CHCl_3$ to 98% $CHCl_3$, 2% methanol over 10 min. After 15 min under isocratic conditions, the methanol concentration was increased over 5 min to 5%. The flow rate was 4 ml/min. [5-^{3}H]NNKOAc eluted at 25 min. The major contaminant in this fraction, [5-^{3}H]4-acetoxy-1-(3-pyridyl)-1-butanone ([5-^{3}H]AcPB), eluted at 23 min. Both compounds were collected and their purities and identities were confirmed by co-elution with standards (9) on reverse-phase HPLC (Whatman Partisil 5 ODS-3 cartridge column, 4.6 mm × 12.5 cm) using a linear gradient from 100% 20 mM sodium phosphate buffer (pH 7) to 50% buffer, 50% of 95% methanol in H_2O over 50 min with a flow rate of 1 ml/min. In order to ensure the radiochemical stability of [5-^{3}H]NNKOAc and [5-^{3}H]AcPB, the crude reaction mixture was maintained at or below −20°C until just prior to HPLC purification. Upon collection from the normal-phase HPLC column, the peaks were immediately concentrated under nitrogen, redissolved in CH_2Cl_2, dried over anhydrous Na_2SO_4, filtered, and stored under nitrogen at −20°C. The overall yield of [5-^{3}H]NNKOAc was 17.5 mCi (3.6%). The sp. act. (90.4 mCi/mmol) was determined by measuring the sp. act. of [5-^{3}H]4-HPB generated from the [5-^{3}H]AcPB isolated from the reaction mixture. [5-^{3}H]NNKOAc was determined to be >90% pure as judged by the reverse-phase HPLC system described above. The main contaminants were [5-^{3}H]4-HPB and [5-^{3}H]AcPB. The purified [5-^{3}H]NNKOAc was stable for several months when stored in dry CH_2Cl_2 at −20°C under nitrogen.

[5-^{3}H]4-Hydroxy-1-(3-pyridyl)-1-butanone ([5-^{3}H]4-HPB). [5-^{3}H]AcPB (3.5 mCi, 38.7 μmol) was hydrolyzed in the presence of esterase (hog liver, Sigma, 0.19 mg) in 20 mM sodium phosphate buffer (pH 7.0) to yield [5-^{3}H]4-HPB. When [5-^{3}H]AcPB was completely consumed, the reaction mixture was extracted with CH_2Cl_2 (10 × 25 ml). The extracts were combined, dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was resuspended in CH_2Cl_2 (5 ml) and stored at −20°C under nitrogen until needed. The identity and radiochemical purity (97%) were judged by co-elution with unlabeled 4-HPB in the reverse-phase system described above. The sp. act. of [5-^{3}H]4-HPB was 90.4 mCi/mmol.

Animal treatments

Female A/J mice (5–6 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME). They were placed on AIN-76A diet at least 1 week prior to the initiation of the experiment and maintained on that diet for the duration of the experiment.

Male F344 rats weighing 270–290 g were obtained from Charles River Breeding Laboratories (Kingston, NY) and were housed under standard conditions as previously described (13).

The mice were given i.p. injections of the appropriate compound in saline. The rats were treated with the saline solutions by gavage. Blood was obtained by cardiac puncture under halothane anesthesia, 24 h post-injection. In the mouse experiments, the lungs and livers were also removed and immediately frozen at −80°C.

Mouse experiments

Groups of four mice (7–9 weeks old) were treated with [5-^{3}H]4-HPB (8.3 μmol, 75 μCi), [5-^{3}H]NNK (10 μmol, 2755 or 1060 μCi), or [5-^{3}H]NNKOAc (6.1 μmol, 550 μCi; 3.0 μmol, 268 μCi; 1.5 μmol, 135 μCi; or 0.58 μmol, 53 μCi). After 24 h, blood was collected by cardiac puncture. The blood from all four animals treated with [5-^{3}H]NNKOAc was pooled, whereas the blood from two animals treated with [5-^{3}H]NNK or [5-^{3}H]4-HPB was pooled. Globin was isolated from the blood as previously described (4). The lungs were removed and pooled. The livers of the [5-^{3}H]NNK- and [5-^{3}H]NNKOAc-treated animals were also removed. DNA was isolated from the tissues using a previously described modification of the Marmur method (5).

Rat experiments

Each of two rats was treated with [5-^{3}H]NNK (0.270 μmol, 55.9 μCi) or [5-^{3}H]4-HPB (0.270 μmol, 44.6 μCi). The blood was collected by cardiac puncture 24 h following exposure. Hemoglobin was isolated from the blood as described (4).

Analysis of DNA for levels of pyridyloxobutylation

The DNA was subjected to strong acid hydrolysis (0.8 N HCl, 6 h, 80°C) as previously described (5). Levels of [5-^{3}H]4-HPB were determined by reverse-phase HPLC analysis with detection by a radioactive flow detector (Flo-one/Beta, Radiomatics Instruments, Tampa, FL). The hydrolysates were neutralized, spiked with standards and separated on a C_{18} reverse-phase column (Whatman Partisil 5 ODS-3 cartridge column, 4.6 mm × 12.5 cm) with solvents A (20 mM sodium phosphate buffer, pH 7) and B (95% methanol, 5% H_2O) using a linear gradient from 100% A to 65% A over 60 min (flow: 1 ml/min). The levels of [5-^{3}H]4-HPB were determined from the radioactivity that co-eluted with the standard. The levels of guanine present in the hydrolysates were determined as previously described (14).

Analysis of globin for levels of pyridyloxobutylation

Hemoglobin was isolated and extensively dialyzed as previously described (4). The globin was precipitated with acidic acetone followed by three acetone washes. A portion of each sample was dissolved by sonication in 2–3 ml of 0.1 N HCl. Monofluor scintillation cocktail (15 ml, National Diagnostics, Manville, NJ) was added and the total amount of binding was determined by scintillation counting. Another portion was sonically dispersed in 0.1 N NaOH for 1 h at room temperature. The resulting mixture was extracted with CH_2Cl_2. The extracts were concentrated and analyzed for 4-HPB by reverse-phase HPLC with radioflow detection as previously described (4). A third portion of the globin was analyzed by reverse-phase HPLC using a large-pore C_4 column (Dynamax-300A 12 μm C_4, 25 cm × 4.6 mm, Rainin Inst. Co., Woburn, MA) linked to a radioflow detector. The column was eluted with solvent A (80% H_2O, 20% acetonitrile, 0.1% trifluoroacetic acid) and solvent B (40% H_2O, 60% acetonitrile, 0.1% trifluoroacetic acid). A linear gradient was employed from 65% A, 35% B to 50% A over 50 min at a flow rate of 1 ml/min. This system separated the α- and β-chains of globin (13). This system was also used to purify the globin from any non-covalently bound metabolites prior to the base treatment described above. The CH_2Cl_2 extracts of these samples were analyzed on normal-phase HPLC (EM LiChrosorb Si-60, 10 μm column, 250 cm × 4.6 mm) using solvents A (100% ethyl acetate) and B (90% ethyl acetate, 10% methanol). A linear gradient from 100% A to 50% A over 20 min was employed. The identity of 4-HPB was confirmed by co-chromatography with 4-HPB standard in this system.

Results

DNA studies

DNA isolated from the lungs of mice treated with [5-^{3}H]NNK, [5-^{3}H]NNKOAc or [5-^{3}H]4-HPB and livers of mice treated with [5-^{3}H]NNK or [5-^{3}H]NNKOAc was hydrolyzed in 0.8 N HCl and analyzed by reverse-phase HPLC chromatography to

determine the levels of [5-^{3}H]4-HPB released from the DNA. The radiograms of the lung DNA hydrolysates from the [5-^{3}H]NNK- and [5-^{3}H]NNKOAc-treated animals and the liver DNA hydrolysate from the [5-^{3}H]NNK-treated animals are illustrated in Figure 2. The hydrolysates of the lung and liver DNA isolated from [5-^{3}H]NNK-treated mice contained several significant radioactive peaks other than [5-^{3}H]4-HPB (Figure 2A,C). The radiograms of the lung DNA hydrolysates differed from those of the liver hydrolysates. These additional peaks were not present in the hydrolysates of [5-^{3}H]NNKOAc-treated mouse lung and liver DNA (Figure 2B). No radioactive peaks were observed in the hydrolysates of lung DNA isolated from the mice treated with [5-^{3}H]4-HPB (data not shown).

The levels of 4-HPB released from lung DNA isolated from [5-^{3}H]NNK-, [5-^{3}H]4-HPB- and [5-^{3}H]NNKOAc-treated mice and liver DNA isolated from [5-^{3}H]NNK- and [5-^{3}H]NNKOAc-treated mice are listed in Table I. Significant amounts of [5-^{3}H]4-HPB were released from lung DNA samples from [5-^{3}H]NNKOAc- and [5-^{3}H]NNK-treated animals. No detectable levels of [5-^{3}H]4-HPB were observed in those from the [5-^{3}H]4-HPB-treated animals. Higher levels of binding of [5-^{3}H]NNKOAc and [5-^{3}H]NNK were observed in liver than in lung. The dose response of adduct formation in the lungs and livers of [5-^{3}H]NNKOAc-treated mice is shown in Figure 3.

Hemoglobin studies

Globin was isolated from the blood of the mice and rats treated with [5-^{3}H]NNK and [5-^{3}H]4-HPB and from mice treated with [5-^{3}H]NNKOAc. The total level of tritium binding to globin was measured. The globin was then treated with 0.1 N NaOH to release 4-HPB. The extracts of these mixtures were analyzed by HPLC in order to determine the levels of [5-^{3}H]4-HPB released from the globin. The results of these studies are listed in Table II.

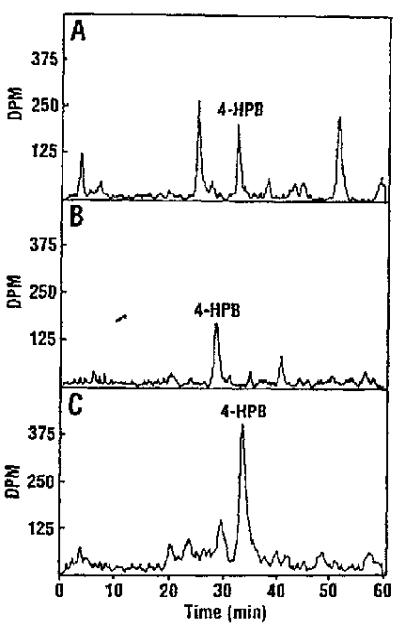

Fig. 2. Representative radiograms obtained upon reverse-phase HPLC analysis of an acid hydrolysate of lung DNA isolated from mice treated with (A) [5-^{3}H]NNK or (B) [5-^{3}H]NNKOAc and (C) liver DNA isolated from mice treated with [5-^{3}H]NNK. In each case, 4-HPB was identified by co-elution with a standard. The retention time of 4-HPB was dependent on the age of the column.

Higher levels of pyridyloxobutylation were observed in the globin of [5-^{3}H]NNK-treated rats than in those given an equimolar dose of [5-^{3}H]4-HPB. In mice, as observed in the DNA studies, levels of total binding and [5-^{3}H]4-HPB released from globin of the [5-^{3}H]NNK- and [5-^{3}H]NNKOAc-treated animals were substantially higher than the corresponding levels in the globin from [5-^{3}H]4-HPB-treated animals. The released 4-HPB represented 14–35% of the total binding of [5-^{3}H]NNKOAc or [5-^{3}H]NNK to globin, whereas no detectable levels of [5-^{3}H]-4-HPB were released from the globin of [5-^{3}H]4-HPB-treated mice or rats.

The globin samples from all treated animals were also analyzed by C_4 HPLC chromatography with radioflow detection. This chromatographic system separates the α- and β-chains of globin. The assignments of the peaks were based on the literature (15). Any free unbound metabolites would elute early in the chromatogram. Typical chromatograms are illustrated in Figure 4. In the globin from [5-^{3}H]NNK- and [5-^{3}H]NNKOAc-treated mice and [5-^{3}H]NNK-treated rats, most of the radioactivity eluted with the UV peak assigned to the β-chain. A higher degree of selectivity was observed in the [5-^{3}H]NNK samples than in the [5-^{3}H]NNKOAc samples. Since the C_4 HPLC system leads to protein denaturation, the radioactivity associated with the protein peaks is covalently bound to protein. When unmodified globin and [5-^{3}H]4-HPB (7000 d.p.m.) were mixed and then

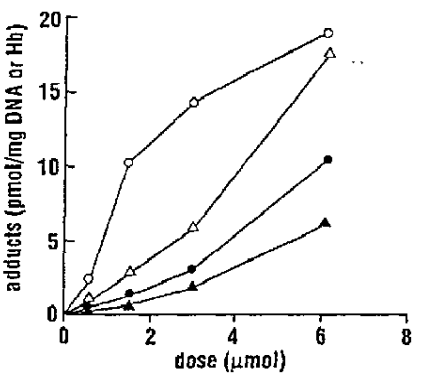

Fig. 3. Dose response for levels of pyridyloxobutylation leading to release of 4-HPB from lung DNA (●—●), liver DNA (○—○), and globin (▲—▲) of mice treated with [5-^{3}H]NNKOAc. Total globin binding in these mice is represented by △—△.

Table I. Levels of [5-^{3}H]4-HPB released from lung and liver DNA of mice treated with [5-^{3}H]NNK, [5-^{3}H]NNKOAc or [5-^{3}H]4-HPB

Treatment[a]	Dose (μmol)	[5-^{3}H]4-HPB released (pmol/μmol guanine)	
		Lung[b]	Liver[c]
[5-^{3}H]NNK	10	8.35	65.3; 62.5
[5-^{3}H]NNKOAc	6.1	15.5	33.7; 22.1
[5-^{3}H]NNKOAc	3.0	4.5	15.5; 26.5
[5-^{3}H]NNKOAc	1.5	(1.8)[d]	11.6; 18.5
[5-^{3}H]NNKOAc	0.6	–	ND; 7.1
[5-^{3}H]4-HPB	8.3	ND	–

[a]Groups of A/J mice were treated with the indicated dose of [5-^{3}H]NNK, [5-^{3}H]NNKOAc or [5-^{3}H]4-HPB in saline (i.p.) and killed after 24 h. DNA was isolated as described in Materials and methods.
[b]DNA was analyzed from a pool of four lungs.
[c]DNA was analyzed from two individual livers.
[d]At limits of detection.
–, not determined; ND, not detected.

Table II. Levels of total binding and [5-^{3}H]4-HPB released from globin of mice or rats treated with [5-^{3}H]NNK, [5-^{3}H]NNKOAc or [5-^{3}H]4-HPB[a]

	Dose (μmol/animal)	Total binding (pmol/mg globin)	[5-^{3}H]4-HPB released[b] (pmol/mg globin)	% of binding as [5-^{3}H]4-HPB released
Mice				
[5-^{3}H]NNK	10	15.2; 15.9[c]	2.3	14.3
[5-^{3}H]NNKOAc[d]	6.1	17.5	6.2	35.2
[5-^{3}H]NNKOAc[d]	3.0	5.9	1.9	31.4
[5-^{3}H]NNKOAc[d]	1.5	2.8	0.69	25.0
[5-^{3}H]NNKOAc[d]	0.6	0.86	0.20	22.9
[5-^{3}H]4-HPB	8.3	1.8; 1.8[c]	ND[e]	–
Rats				
[5-^{3}H]NNK	0.27	0.16	0.03	19.2
[5-^{3}H]4-HPB	0.27	0.026	ND[f]	–

[a]Groups of four A/J mice were treated with the indicated doses of [5-^{3}H]NNK, [5-^{3}H]NNKOAc or [5-^{3}H]4-HPB in saline (i.p.) and killed after 24 h. Two rats were each treated with the indicated doses of [5-^{3}H]NNK or [5-^{3}H]4-HPB by gavage and killed after 24 h. Globin was isolated as described in Materials and methods.
[b]4-HPB was released from globin upon base treatment.
[c]Results were obtained from two samples of blood from two mice.
[d]Results were obtained from the combined blood of four mice.
[e]ND, not detected; limits of detection were 0.09 pmol/mg globin.
[f]Limits of detection were 0.009 pmol/mg globin.

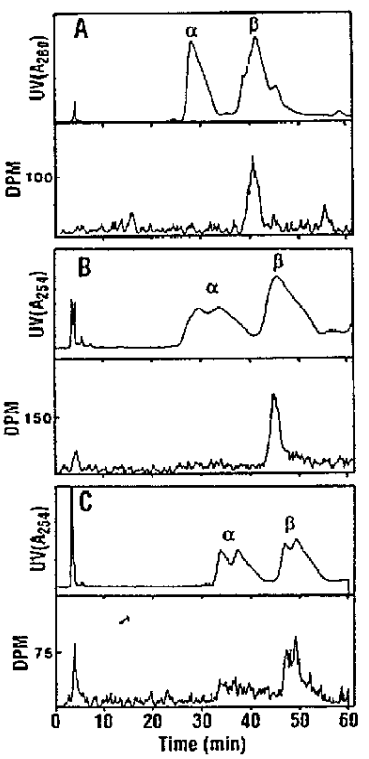

Fig. 4. Radiochromatograms obtained upon reverse-phase HPLC analysis of globin isolated from (A) a rat treated with [5-^{3}H]NNK, (B) mice treated with [5-^{3}H]NNK and (C) mice treated with [5-^{3}H]NNKOAc.

analyzed by the C_4 chromatographic system, all of the radioactivity eluted between 2 and 3 min, whereas the globin eluted after 25 min, demonstrating that [5-^{3}H]4-HPB does not bind to globin *in vitro*. No radioactivity was detected in the C_4 chromatogram of the globin from [5-^{3}H]4-HPB-treated mice (data not shown).

The C_4 column was also used to purify the globin prior to base treatment to ensure that there were no free unbound metabolites associated with it. The fractions containing both the α- and β-chains were pooled and then submitted to base treatment. The methylene chloride extracts of the base-treated globin of the [5-^{3}H]NNK-treated rats and mice and the [5-^{3}H]NNKOAc-treated mice were analyzed by normal-phase HPLC chromatography with radioflow detection to determine the extent of [5-^{3}H]4-HPB released and also to confirm that we were measuring 4-HPB and not 3-HPB. These two alcohols co-elute under the reverse-phase HPLC conditions. 4-HPB, not 3-HPB, was observed in all globin samples except that from the [5-^{3}H]4-HPB-treated animals. No detectable levels of either 3-HPB or 4-HPB were observed in those samples.

Discussion

These studies clearly demonstrate that NNK is activated by methyl hydroxylation to a reactive species that is capable of reacting with cellular nucleophiles in DNA and protein. It is this reactive species, and not 4-HPB, that forms DNA and hemoglobin adducts that can be hydrolyzed to 4-HPB. This conclusion is supported by the observation of much higher levels of DNA and hemoglobin binding in the [5-^{3}H]NNK- and [5-^{3}H]NNKOAc-treated animals than in the [5-^{3}H]4-HPB-treated animals at comparable doses. The protected hydroxymethyl metabolite, NNKOAc, in the presence of esterases, is hydrolyzed to 4-(hydroxymethylnitrosamino)-1-(3-pyridyl)-1-butanone, the proposed hydroxymethyl metabolite of NNK.

The observation of several other radioactive peaks in the chromatogram of the acid hydrolysates of [5-^{3}H]NNK-treated lung and liver DNA suggests that mouse liver and lung have activation routes in addition to methyl hydroxylation. These alternative pathways do not appear to occur in rats since the radiograms of acid hydrolysates of liver DNA isolated from [5-^{3}H]NNK-treated rats did not display these extra peaks (5). The source and chemical nature of the new radioactive peaks in the mouse lung and liver DNA hydrolysates is not known but is currently under investigation. One potential bioactivation pathway for NNK involves reduction to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), the major *in vivo* metabolite of NNK (16,17), followed by activation to a DNA or hemoglobin binding species upon α-hydroxylation.

The binding of the pyridyl portion of NNK and NNKOAc to

hemoglobin was surprisingly selective, presumably for the β chain (Figure 4). The binding of NNK was more selective than NNKOAc. Such striking selectivity for the β-chain has also been observed with 4-aminobiphenyl (18,19) and 4-dimethylaminophenol (20). Both of these compounds are bound exclusively to the β-cysteine-93. The specificity of these alkylation reactions is probably due to catalysis by the hemoprotein of the final activation reaction leading to globin modification. The major fluoranthene–hemoglobin adduct in rats results from the binding of reactive fluoranthene 2,3-dihydrodiol-1,10b-epoxide to the β-cysteine-125 (21). It is unlikely that high levels of pyridyloxobutylation of β-cysteinyl residues occur since NNK–cysteine adducts were not detected in hemoglobin (22). The mechanism of *in vivo* globin adduct formation from NNK and NNKOAc is unknown. Nor is it known where the reactive species that generates these adducts is formed. Further investigations into these questions are warranted.

While the binding of [5-^{3}H]NNK and [5-^{3}H]NNKOAc to DNA and globin was significantly higher than observed with [5-^{3}H]4-HPB, some radioactivity is associated with globin but not DNA when [5-^{3}H]4-HPB is given *in vivo* to both mice and rats. However, no detectable radioactivity was released as 4-HPB. 4-HPB was previously found to be unreactive with DNA (5) and hemoglobin (4) *in vitro*. These results suggest that, while 4-HPB appears to bind to hemoglobin *in vivo* via some unknown mechanism, the adducts do not release 4-HPB under the hydrolysis conditions. Therefore the levels of 4-HPB released from hemoglobin exposed to NNK *in vivo* can be attributed to the bioactivation of NNK to a reactive intermediate that alkylates hemoglobin and DNA, not to the initial formation of 4-HPB followed by its reaction with these nucleophiles. Thus the release of 4-HPB from DNA and hemoglobin isolated from tobacco users can be used as a measure of the individual's ability to activate tobacco-specific nitrosamines to a reactive species.

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